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Detection of a Novel DNA Virus (TTV) Sequence in Peripheral Blood Mononuclear Cells

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DNA sequences of a novel DNA virus (TTV) were examined in 81 peripheral blood mononuclear cell (PBMC) DNA samples from 48 children and 33 adults, 22 cord blood mononuclear cells (CBMC) DNA samples, and 7 autopsy liver tissue DNA samples by a hemi-nested polymerase chain reaction (PCR). The PCR was carried out using the published primers (NG059, NG061, NG063) to amplify TTV DNA sequences. The sequences were detected in 4 of 81 (5%) PBMC DNA samples, in none of 22 (0%) CBMC DNA samples, and in 2 of 7 (29%) liver tissue DNA samples by direct gel analysis. The PCR-amplified products were confirmed by direct sequencing. The sequencing showed considerable diversities, with differences of 0–55% in 6 TTV isolates, compared with the prototype sequence of TTV. These results suggest that TTV is a ubiquitous virus that produces asymptomatic infection in a large proportion of the general population without transfusion of blood-derived products. To our knowledge, this is the first report describing the detection of TTV DNA sequences in PBMCs. ***J. Med. Virol. 58:174–177, 1999.***

KEY WORDS: TTV; posttransfusion hepatitis; peripheral blood mononuclear cells

INTRODUCTION

Recently, Nishizawa et al. [1997] and Okamoto et al. [1998] identified a novel DNA clone (N22) from serum of a patient (TT) with posttransfusion non-A to non-G hepatitis by the method of representational difference analysis. DNA sequences of a novel virus, named TTV, were detected in sera from 9 of 19 (47%) patients with fulminant hepatitis and 41 of 90 (46%) patients with chronic liver disease of unknown etiology. These results suggested that TTV would be responsible for a portion of fulminant and chronic liver disease of unknown etiology. However, TTV DNA sequences were also detected in sera from 12% of blood donors. Little is known about primary infection with TTV and sites of viral persistence and reactivation after infection in the host. The purpose of this study was to determine whether TTV DNA sequences are present in peripheral blood mononuclear cells (PBMCs), cord blood mononuclear cells (CBMCs), and liver tissue.

MATERIALS AND METHODS

DNA Samples

Eighty-one PBMC DNA samples from 48 children and 33 adults, 22 CBMC DNA samples, and 7 autopsy liver tissue DNA samples were examined by a hemi-nested polymerase chain reaction (PCR) procedure. Twenty-five children with acute febrile illnesses (aged 2 months to 6 years), 10 patients with Kawasaki disease (aged 2 months to 5 years), and 13 patients with infectious mononucleosis (aged 2–12 years) were examined. Acute febrile illnesses included mainly bacterial respiratory infections. Adults consisted of 13 healthy adults (aged 25–40 years), 12 renal transplant patients (aged 23–41 years), and 8 pregnant women (aged 24–32 years). The renal transplant patients were treated with basic immunosuppressive therapy after transplantation. Autopsy liver tissue DNA samples were obtained from 7 patients (aged 2–22 years) with chronic active Epstein-Barr virus (EBV) infection [Kikuta et al., 1988].

PBMCs and CBMCs were obtained by centrifugation of heparinized blood on a Ficoll-Paque gradient. The genomic DNA was extracted from these cells and tissue blocks by digestion with proteinase K, extraction with phenol-chloroform, and precipitation with ethanol.

PCR

The first-round PCR conditions were as follows: 94°C for 10 min followed by 35 cycles of 94°C for 30 sec, 60°C

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TABLE I. Prevalence of TTV DNA Sequences

Sample	Detected/total (%)
Peripheral blood mononuclear cells	4/81 (4.9)
Children	3/48 (6.2)
Acute febrile illnesses	2/25 (8.0)
Kawasaki disease	1/10 (10.0)
Infectious mononucleosis	0/13 (0)
Adults	1/33 (3.0)
Healthy	0/13 (0)
Renal transplant	0/12 (0)
Pregnant	1/8 (12.5)
Cord blood mononuclear cells	0/22 (0)
Liver	2/7 (28.5)

for 45 sec and 72°C for 45 sec. The reactions were terminated by a 7-min extension at 72°C. The PCR reaction mixture consisted of 200 μmol of each deoxyribonucleotide, 2.5 U of AmpliTaq Gold, 50 nmol/l of potassium chloride, 10 mmol/l of Tris-HCl (pH 8.3), 1.5 mmol/l of magnesium chloride, 0.01% (wt/vol) of gelatin, 20 pmol of each primer, and approximately 1 μg of DNA in a volume of 100 μl. The sequences of the first-round PCR primers were 5′ACAGACAGAGGAGAAGGCAACATG3′(NG059; nucleotide position 1900–1922) and 5′CTGGCATTTTACCATTTCCAAAGTT3′ (NG063; nucleotide position 2161–2185) [Okamoto et al., 1998]. After the first-round PCR, 10 μl of PCR solution was added to 90 μl of second-round PCR mixture. The second-round amplification was carried out as in the first round, except for 25 cycles and primer pair. The sequences of the second-round PCR primers were NG063 and 5′GGCAACATGTTATGGATAGACTGG3′ (NG061; nucleotide position 1915–1938) [Okamoto et al., 1998]. Aliquots (10 μl) of the PCR-amplified product were subjected to electrophoresis through a 2.0% agarose gel. The gel was stained with ethidium bromide for 5 min, and the DNA bands were visualized under ultraviolet light.

Sequencing

The PCR-amplified products were sequenced directly by using an ABI PRISM™ Dye terminator cycle sequencing ready reaction kit (Perkin Elmer) with an ABI 310 Genetic Analyzer (Applied Biosystems). Both sense and antisense strands of the PCR-amplified products were sequenced directly.

RESULTS

Direct Gel Analysis of PCR-Amplified Products

Table I summarizes the prevalence of TTV DNA sequences. The TTV DNA sequences, as indicated by the 271 base-pair DNA band in Figure 1, were detected in PBMCs from 3 of 48 children. The 3 children included 2 patients (aged 1 year and 5 years) with acute febrile illnesses and 1 patient (aged 5 years) with Kawasaki disease. They had not received any blood-derived products. Only one PBMC DNA sample of a pregnant woman (aged 32 years) was PCR-positive among PBMCs DNA samples from 33 adults. (She also did not receive any blood-derived products.) All of the CBMC DNA samples were PCR-negative for the sequences. Two (aged 4 and 18 years) of seven liver tissue DNA samples from patients with chronic active EBV infection were PCR-positive for the sequences.

Sequencing

The results of the sequence analysis are summarized in Figure 2. When the sequence of 222 bases (nucleotide position 1939–2160) was compared with N22 clone of TTV, only one DNA sample from a patient with chronic active EBV infection showed the same sequence as the prototype sequence of TTV [Nishizawa et al., 1997; Okamoto et al., 1998]. Another DNA sample from a pregnant woman had a single-base substitution. However, all the other DNA samples showed considerable diversities in sequence, with differences of 0–55% compared with the N22 clone of TTV.

DISCUSSION

It has been reported that TTV DNA sequences are detectable more frequently in patients with liver diseases of unknown etiology than in blood donors. Furthermore, TTV DNA titers are reported to be closely associated with elevated transaminase levels in sera from the patients with the liver disease and the sequences are detected in their liver tissues in titers equal to or 10–100 times higher than those in the corresponding sera. These findings indicate that TTV could be responsible for a portion of liver diseases of unknown etiology [Nishizawa et al., 1997; Okamoto et al., 1998]. The TTV genome is thought to be an unenveloped, single-stranded DNA molecule, which is at least 3739 bases long [Nishizawa et al., 1997; Okamoto et al., 1998]. Of known human unenveloped, single-stranded DNA viruses, there are two parvoviruses, parvovirus B19 and adeno-associated viruses (AAV). Although TTV has been shown to have no similarity in amino acid sequences to either parvovirus, the constitution of TTV genome and two open reading frames show several points of resemblance [Nishizawa et al., 1997; Okamoto et al., 1998]. Most of the adult human population is seropositive for parvovirus B19 and AAV, and seroconversion occurs most frequently in children and young adults [Berns and Bohenzky, 1987; Heegaard and Hornsleth, 1995]. Although an association of AAV with any disease or pathological condition has not yet been determined [Berns and Bohenzky, 1987], parvovirus B19 is associated with several clinical syndromes, including erythema infectiosum, hydrops fetalis, aplastic crises in patients with hemolytic anemia, and chronic anemia due to persistent infection in immunocompromised host [Heegaard and Hornsleth, 1995]. The transmission of parvovirus B19 is known to occur through respiratory secretions and transfusion of blood-derived products, and from mother to fetus vertically [Heegaard and Hornsleth, 1995].

The number of samples in present study was too small to permit a strong statistical significance. Nevertheless, the presence of TTV DNA sequences in

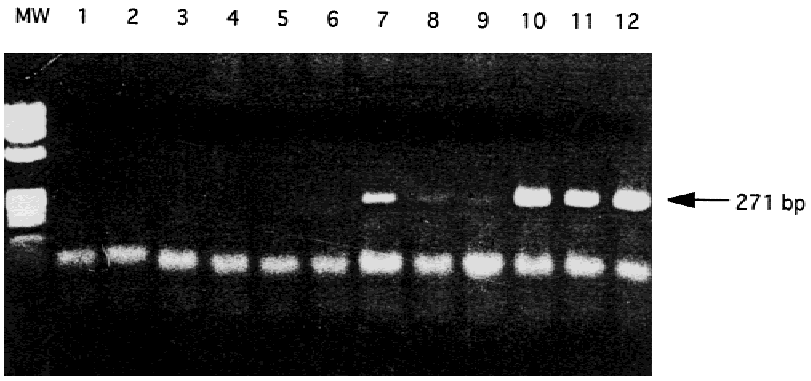

Fig. 1. Ethidium bromide staining of polymerase chain reaction (PCR)-amplified products. The DNA used for PCR was as follows: **Lanes 1–6,** cord blood mononuclear cell (CBMC) DNA. **Lane 7,** peripheral blood mononuclear cell (PBMC) DNA from pregnant woman. **Lanes 8** and **9,** PBMC DNA from patients with acute febrile illnesses. Lane 10, PBMC DNA from patient with Kawasaki disease. Lanes 11 and 12, liver DNA from patients with chronic active Epstein-Barr virus infection. MW, molecular weight marker: ϕx174/*Hae*III digest.

	1939								GGCAAC	ATGTTATGGA
N22	TAGACTGGCT	AAGCAAAAAA	AACATGAACT	ATGACAAAGT	ACAAAGTAAA	TGCTTAATAT	CAGACCTACC	TCTATGGGCA	GCAGCATATG	
1	--	----------	----------	----------	----------	----------	----------	----------	----------	
2	--	--CT---G-T	-G-TCAG-A-	-CTCA---AC	---G--C--G	--TC-----G	A-A--A-G--	CT-G-----C	T---T---C-	
3	--	--CT---G-T	-C-TCAG-A-	-CTCA---AC	---G--C--G	--TC-----G	A-A--A-G--	CT-G-----C	T---T---C-	
4	TG	CTCT---G-C	G---CC-C--	T-CGAG-TC-	--C-CAG-G-	-ATGCTG--A	A----A-T--	-T--------	---TTTATG-	
5	--	--CT---G-T	-G-TCAG-A-	-CTCA---AC	---G--C--G	--CT-----G	A-A--A-G--	CT-G-----C	T---T---C-	
6	--	----------	----------	----------	----------	----------	----------	----------	----------	
N22 2021	GATATGTAGA	ATTTTGTGCA	AAAAGTACAG	GAGACCAAAA	CATACACATG	AATGCCAGGC	TACTAATAAG	AAGTCCCTTT	ACAGACCCAC	
1	----------	----------	----------	----------	----------	----------	----------	----------	----------	
2	----CAC---	G-AC--CAGC	---GTA----	-----AC---	----G-ACAC	--CTGT--AT	GTG-T--T--	---C----A-	----TA----	
3	----CAC---	G-AC---CAG	C---------	-----AC---	----G-ACAC	--CTGT--AT	GTG-T--T--	---C----A-	----TA----	
4	-C--CAG---	CT-TGT--CT	--GGC-CTCC	AC----CCGG	AC-CAGT-AA	G-G-T---AG	--ACT--T-T	-T-C--A-A-	---A-A--CA	
5	----CAC---	G-AC--CAGC	---GTA----	-----AC---	----G-AGAC	--CTGT--AT	GTG-T--T--	---C----A-	----TA----	
6	----------	----------	----------	----------	----------	----------	----------	----------	----------	
							NG063			
N22 2111	AACTACTAGT	ACACACAGAC	CCCACAAAAG	GCTTTGTTCC	TTACTCTTTA	AACTTTGGAA	ATGGTAAAAT	GCCAG		
1	----------	----------	-----G----	----------	----------					
2	----GT---A	C----ACA-T	---CTC-G--	-A-A---A--	---TAGC--C					
3	----GT----	C----ACA-T	---CT--G--	-A-A---A--	---TAGC--C					
4	-G--CTACAA	A-CAGACAG-	A-AGACG---	---AC--A--	C--TGAC-AT					
5	----GT---A	C------AT-	---T---G--	-A-A---A--	---TAGC--C					
6	----------	----------	----------	----------	----------					

Fig. 2. Comparison of nucleotide sequences of TTV isolates. 1, peripheral blood mononuclear cell (PBMC) DNA from pregnant woman. 2 and 3, PBMC DNA from patients with acute febrile illnesses. 4, PBMC DNA from patient with Kawasaki disease. 5 and 6, liver DNA from patients with chronic active Epstein-Barr virus infection. Hyphens indicate the same nucleotide as in N22. NG061 and NG063 were used as primers in the second-round polymerase chain reaction.

PBMCs from three children and one adult without transfusion of blood-derived products suggested that TTV might be a ubiquitous agent that frequently infects individuals of all ages without inducing clinically evident disease, possibly except for posttransfusion hepatitis. The frequency of positive samples among children seemed to be higher than that among adults. TTV DNA sequences were not detected in 22 CBMC DNA samples. Furthermore, the corresponding CBMC DNA sample, whose mother's PBMCs contained TTV DNA sequences, was PCR-negative for the sequences, and thus there was no evidence that the route of transmission of TTV was intrauterine or perinatal infection from mother to fetus. Thus, although the routes of transmission of TTV have not yet been clearly identified, three children and one adult have not received any blood-derived products, indicating that TTV appears to be transmitted more frequently via routes other than transfusion of blood-derived products. TTV DNA sequences were not detected in PBMC DNA samples from 12 renal transplant patients, indicating that active replication of the virus might not occur during immunosuppression.

Stored DNA samples were used in this study so that the prevalence of TTV DNA sequences in PBMCs could not be compared with that in corresponding sera. Okamoto et al. [1998] reported that the prevalence of TTV DNA sequences was 12% in sera from blood donors. On the other hand, the prevalence of the sequences was 3% in PBMC samples from adults in this study. Taken

together, it is conceivable that the frequency of virus detection in PBMCs might be much lower than that in sera. TTV DNA sequences were detected frequently in liver tissue DNA samples (28%) from the patients with chronic active EBV infection. However, as there was no liver tissue as control, the association between TTV infection and chronic active EBV infection could not be examined.

Well-conserved regions of TTV were used as oligonucleotide primers for the detection of TTV DNA. However, TTV DNA in PCR-amplified products in the present study had considerable sequence variability, indicating that the primers used by the hemi-nested PCR could not amplify all TTV DNA. The absolute prevalence of TTV infection cannot yet be determined. A serological test is essential for determination of the distribution of the infection in the human population and for identifying the full spectrum of disease states associated with TTV. Further studies are required to elucidate natural infection with TTV.

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